Vaccination-induced skin-resident memory CD8$^+$ T cells mediate strong protection against cutaneous melanoma


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ABSTRACT
Memory CD8+ T cell responses have the potential to mediate long-lasting protection against cancers. Resident memory CD8+ T (Trm) cells stably reside in non-lymphoid tissues and mediate superior innate and adaptive immunity against pathogens. Emerging evidence indicates that Trm cells develop in human solid cancers and play a key role in controlling tumor growth. However, the specific contribution of Trm cells to anti-tumor immunity is incompletely understood. Moreover, clinically applicable vaccination strategies that efficiently establish Trm cell responses remain largely unexplored and are expected to strongly protect against tumors. Here we demonstrate that a single intradermal administration of gene- or protein-based vaccines efficiently induces specific Trm cell responses against models of tumor-specific and -antigens, which accumulated in vaccinated and distant non-vaccinated skin. Vaccination-induced Trm cells were largely resistant to in vivo intravascular staining and antibody-dependent depletion. Intradermal, but not intraperitoneal vaccination, generated memory precursor expressing skin-homing molecules in circulation and Trm cells in skin. Interestingly, vaccination-induced Trm cell responses strongly suppressed the growth of B16F10 melanoma, independently of circulating memory CD8+ T cells, and were able to infiltrate tumors. This work highlights the therapeutic potential of vaccination-induced Trm cell responses to achieve potent protection against skin malignancies.

Introduction
Immunotherapy is emerging as a new form to treat cancer by harnessing the activity of cytotoxic CD8+ T lymphocytes (CTLs) that specifically recognize tumor-associated antigens. Transfusion of autologous tumor-specific CTLs have demonstrated to elicit durable clinical benefit in a significant proportion of patients with melanoma, leukemia, lymphoma and other cancers, who failed to respond to conventional treatments. Vaccination strategies eliciting CTL responses specific for tumor-specific and -antigens have shown promising results in recent clinical trials. Long-lasting protective immunity relies on the efficient establishment of long-lived memory CD8+ T cells, which have the potential to eradicate primary and disseminated tumors. They have been typically classified in two subsets: effector-memory (Tem) and central-memory (Tcm) CD8+ T cells. Tcm cells express high levels of both CD62L and CCR7, which enable them to recirculate between blood and secondary lymphoid organs. On the contrary, Tem cells lack the expression of CD62L and CCR7, but express tissue-homing receptors that enable them to recirculate through the blood and non-lymphoid tissues. Tcm cells have been shown to mediate more potent anti-tumor immunity than Tem cells, due to their enhanced ability to proliferate upon antigen re-encounter.

In addition to circulating Tem and Tcm subsets, resident-memory CD8+ T (Trm) cells stably reside in non-lymphoid tissues, such as skin, lung, intestine, brain, female reproductive tract, and others. Trm cells do not express CD62L or CCR7 and constitutively up-regulate CD69 and integrin αE (CD103)/β7 (commonly referred to as CD103), which are responsible for avoiding tissue egress and facilitating interaction with epithelial barriers, respectively. Trm cells are long-lived and represent the first line of defense against pathogen reinfection. After antigen recognition, Trm cells undergo a rapid and potent activation, characterized by the production of high amounts of effector molecules, such as IFN-γ, TNF-α,
and granzyme B, as well as proinflammatory cytokines, chemokines and antimicrobial molecules.\textsuperscript{19} As a result, Trm cells trigger an innate-like alarm state that can protect against antigenically unrelated pathogens and recruit other immune cells to the site of infection, such as circulating memory T cells and B cells.\textsuperscript{19,20} The remarkable ability of Trm cells to mediate protective adaptive and innate immune responses has attracted the attention for developing vaccination strategies that exploit the ability of Trm cells to prevent infectious diseases.\textsuperscript{21,22}

Although most of studies on Trm cells have been performed in the context of viral infections, studies analyzing the infiltration of CD8\textsuperscript{+} T cells in human solid tumors have shed some light on the possible role of Trm cells in anti-tumor immunity. Tumor infiltration of CD8\textsuperscript{+} T cells displaying a Trm phenotype, mainly based on CD103 expression, was shown to predict a more favorable prognosis in patients with ovarian, endometrial, breast and lung cancers.\textsuperscript{23-28} One of these studies showed that Trm cells infiltrating lung tumors exhibited cytotoxic activity against autologous tumor cells after blocking inhibitory receptors.\textsuperscript{27} CD103 enables tissue residency and additionally, has been shown to promote anti-tumor cytolytic activity of CD8\textsuperscript{+} T cells.\textsuperscript{27,29,30} In melanoma, metastatic lesions contain a significant proportion of tumor-infiltrating Trm cells with CD69\textsuperscript{+}CD103\textsuperscript{+} and CD69\textsuperscript{+}CD103\textsuperscript{+} phenotypes.\textsuperscript{31} Interestingly, a recent report indicated that acquisition of a distinct transcriptional program is required for T cell residency in both tumors and non-lymphoid tissues.\textsuperscript{32}

A causal contribution of Trm cells against melanoma has been recently depicted from mouse models. Results indicated that Trm cells generated by a model of autoimmune vitiligo inhibit the development of melanoma tumors in a CD103-dependent manner.\textsuperscript{33} More recent reports evidenced a cooperative role of Trm and Tcm cells, since cutaneous infection with recombinant ovalbumin (OVA)-expressing vaccinia virus generated Trm cells and delayed growth of melanoma tumors expressing full-length OVA.\textsuperscript{34} Although encouraging, this study did not rule out the potential contribution of vaccinia virus-induced OVA-specific CD4\textsuperscript{+} T cells,\textsuperscript{35} which have been previously shown to acquire anti-tumor activity.\textsuperscript{36} Therefore, the contribution of the vaccination-induced Trm cell compartment against melanoma has not been completely understood yet. Moreover, clinically applicable strategies that efficiently establish Trm cell responses need to be described and are expected to have a big impact in cancer immunotherapy.

Here, we demonstrated that intraderal administration of clinically relevant vaccines efficiently induces Trm cells specific for tumor-specific and self-antigens that accumulate in vaccinated and non-vaccinated skin. Interestingly, vaccination-induced Trm cells strongly suppress the growth of melanoma, independently of circulating CD8\textsuperscript{+} T cells, and were able to infiltrate melanoma tumors. Therefore, our work highlights the therapeutic potential of vaccination-induced Trm cells to achieve potent protection against skin malignancies.

## Results

We first sought to identify clinically applicable vaccination strategies that efficiently generate Trm cell responses in skin. We tested intradermal vaccination using two types of vaccines known to induce strong CD8\textsuperscript{+} T cell responses: DNA vaccines assisted by electroporation\textsuperscript{37,38,39},\textsuperscript{40} and dendritic cell-targeted protein vaccines, using anti-DEC-205 antibodies.\textsuperscript{39,40} Initially, we vaccinated mice with a DNA vaccine encoding ovalbumin (DNA-OVA) as a model of tumor-specific non-self antigen. Prior to vaccination, mice were transferred with naïve CD8\textsuperscript{+} T cells from T cell receptor (TCR) transgenic OTI mice, which recognize the OVA\textsubscript{257-264} epitope. Effector and memory responses were analyzed two and four-five weeks later, respectively. We observed that DNA vaccination efficiently generated antigen-specific CD8\textsuperscript{+} T effector (Teff) cells (Fig. 1a), which predominantly displayed a memory precursor phenotype characterized by low expression of KLRG1 and higher expression of IL-7 receptor (CD127) (CD127) (Fig. 1b, left panel). These memory precursors expressed CXCR3, as well as the skin homing molecules E- and P-selectin ligands (ESL and PSL, respectively) (Fig. 1b), which are important for efficient establishment of Trm cells in the skin.\textsuperscript{18} Of note, CD45.1\textsuperscript{+} CD8\textsuperscript{+} T cells produced IFN-\gamma after ex vivo OVA\textsubscript{257-264} peptide stimulation, while CD45.1\textsuperscript{+} CD8\textsuperscript{+} T cells did not (data not shown). This indicates that only transferred OTI CD8\textsuperscript{+} T cells became expanded after vaccination, outcompeting the endogenous repertoire, as demonstrated by other authors.\textsuperscript{19} At the memory phase, we detected antigen-specific Trm cells defined by the co-expression of CD69 and CD103 in vaccinated skin and, interestingly, also in distant non-vaccinated skin (Fig. 1c-d). This could be a result of skin-wide seeding of Trm cell precursors at the effector phase of the response,\textsuperscript{16,32,41} and subsequent dissemination through the epidermis.\textsuperscript{42} Additionally, a significant proportion of CD69\textsuperscript{+}CD103\textsuperscript{-} OVA-specific CD8\textsuperscript{+} T cells were present in vaccinated skin (Fig. 1d), that may correspond to inflammation-driven Trm cells, which have been described to accumulate at the site of infection.\textsuperscript{43} We next tested a protein-based vaccine that specifically delivers antigen to cross-presenting dendritic cells (DCs) by fusing OVA protein to a DEC-205-specific antibody (\alpha DEC-OVA).\textsuperscript{44} Similar to the DNA vaccine, intraderal vaccination with \alpha DEC-OVA, in combination with poly(I:C) as adjuvant (Protein-OVA), efficiently generated Teff cells (Fig. 1a), as well as Trm cells lodged in both vaccinated and distant skin (Fig. 1d, lower panels). In contrast to DNA vaccination, \alpha DEC-OVA did not induce a significant accumulation of CD69\textsuperscript{+}CD103\textsuperscript{-} OVA-specific CD8\textsuperscript{+} T cells in the vaccinated site. As expected, vaccination-induced Trm cells displayed elevated expression of CD44, PD-1 and CD127 (Fig. 1e).

To demonstrate the residency of OVA-specific CD8\textsuperscript{+} T cells found in the skin, we carried out intravascular staining\textsuperscript{45} and showed that vaccination-induced OVA-specific CD8\textsuperscript{+} T cells were largely refractory to CD8\beta staining, and positive for CD69 and CD103 expression (Fig. 2a). In contrast, antigen-specific memory CD8\textsuperscript{+} T cells found in other tissues, such as lungs, were positive for CD8\beta staining and lacked expression of CD69 and CD103 (Fig. 2a), indicating that they derived from circulation. Since previous publications have reported that skin Trm cells are resistant to antibody-dependent depletion in mice and humans,\textsuperscript{46,47} we treated mice with an anti-CD8 (\alpha CD8)-depleting antibody four weeks after vaccination, at the memory phase of the response, to eliminate all circulating CD8\textsuperscript{+} T cells, while sparing Trm cells. As a control, mice were
treated with isotype-matched control (αCTRL) antibody. As expected, αCD8 administration efficiently depleted total CD8+ T cells in peripheral tissues such as lungs, spleen and lymph nodes (Supplementary Figure 1a). As also anticipated, CD69+CD103−OVA-specific skin Trm cells were largely resistant to antibody-dependent depletion (Fig. 2b). In opposition, OVA-specific circulating memory CD8+ T cells were efficiently depleted from spleen and lungs (Fig. 2c). It is worth mentioning that DNA vaccine-induced CD69+CD103−OVA-specific CD8+ T cells present in vaccinated skin resulted positive for CD8β staining and were depleted after αCD8 administration (data not shown), indicating that they are in equilibrium with the circulation. Overall, these results demonstrate that vaccination-induced skin Trm cells share distinctive characteristics broadly attributed to tissue residency.

We next sought to dissect the contribution of the Trm cell compartment to anti-melanoma immunity in mice bearing vaccination-induced skin Trm cell responses, but depleted of circulating CD8+ T cells. Although the S1PR1 antagonist FTY720 has been broadly employed for restraining circulating CD8+ T cells in lymphoid organs and inhibiting their migration to peripheral tissues, we observed that CD8+ T cell depletion is far more efficient than FTY720 at reducing circulating CD8+ T cell numbers in peripheral blood (Supplementary Figure 1b). Moreover, evidence indicates that FTY720 may affect tumor growth by directly suppressing tumor cell viability, impairing T cell function or interfering with the ability of T cells to infiltrate tumors. As schematized in Fig. 3a, vaccinated mice were treated with αCD8 antibody four weeks after vaccination and one week later, intradermally challenged with B16F10 melanoma cells expressing full-length OVA (B16F10-OVA). DNA and protein-based vaccination completely abrogated the development of otherwise fast-growing melanoma cells injected in the flank close to the vaccinated skin of αCD8-treated mice (Fig. 3b). To discard the contribution of other arms of the adaptive immunity that are induced with vaccines comprising full-length OVA, vaccinated mice were challenged with B16F10 melanoma cells expressing H-2Kb-restricted OVA (B16F10-OTI). These cells exhibited similar tumor growth kinetics compared to B16F10-OVA and parental B16F10 cells in non-treated mice (Supplementary Figure 2a). In this setting, we also observed full protection in...
OVA-vaccinated mice (Fig. 3c), indicating that depletion-resistant Trm cell responses are sufficient to provide strong anti-tumor immunity, independently of circulating CD8<sup>+</sup> T cells.

Anti-tumor protection was antigen-specific since mice bearing OVA-specific Trm cells were not protected against control B16F10 cells (Supplementary Figure 2b). Remarkably, OVA-vaccinated mice were also fully protected against B16F10-OVA or B16F10-OTI cells injected in skin distant from the vaccination site (Fig. 3d), suggesting that the vaccination-induced Trm cell compartment mediates strong and skin-wide protection against melanoma.

To establish the potential of intradermal vaccination to elicit protective Trm cell responses from the endogenous T cell repertoire, which is closer to a clinical setting, DNA vaccination was carried out in mice that did not receive adoptive transfer of TCR-transgenic OTI CD8<sup>+</sup> T cells. Two weeks after vaccination, OVA-specific Teff cells were detected in blood (Fig. 4a), although at lower magnitude as compared with the previous setting using OTI CD8<sup>+</sup> T cells. At the memory phase, comparable OVA-specific Trm cell responses were generated in vaccinated and distant skin (Fig. 4b). More importantly, mice bearing OVA-specific Trm cells raised from the endogenous repertoire suppressed the formation of melanoma tumors in skin close to the vaccination site and also at distant sites (Fig. 4c). These results indicate that Trm cell responses induced from the endogenous repertoire can also mediate potent skin-wide protection against melanoma.

To confirm that αCD8 depletion abrogates protective circulating memory, we then delivered the αDEC-OVA vaccine intraperitoneally, attempting to avoid the generation of resident memory, while maintaining circulating memory responses. Both intradermal and intraperitoneal immunizations yielded similar levels of OVA-specific Teff cell responses (Fig. 5a). However, intradermal, but not intraperitoneal, vaccination led to the generation of specific memory precursors displaying the skin-homing molecules PSL and ESL (Fig. 5b). As anticipated, intradermal and intraperitoneal vaccinations generated similar levels of circulating memory CD8<sup>+</sup> T cells (Fig. 5c), but only intradermal vaccination efficiently generated specific Trm cells in skin (Fig. 5d). Interestingly, both vaccination routes led to protection against intradermal challenge with B16F10-OVA in non-depleted αCTRL-treated mice (Fig. 5e, left panel). As expected, tumor protection was severely impaired in intraperitoneally vaccinated mice devoid of circulating CD8<sup>+</sup> T cells, and all these mice succumbed to melanoma challenge (Fig. 5e, right panel). In contrast, intradermally vaccinated mice...
Vaccination-induced skin Trm cells mediate strong protection against cutaneous melanoma independently of circulating CD8⁺ T cells. C57 BL/6 mice were intravenously transferred with OVA-specific CD45.1⁺ OTI CD8⁺ T cells and the day later, were intradermally vaccinated with DNA-OVA or Protein-OVA. Control mice (CTRL) were vaccinated with empty plasmid (for DNA vaccination) or left unvaccinated (for protein vaccination). After 4–5 weeks, mice were depleted of circulating CD8⁺ T cells by administering anti-CD8 antibody and one week later, were intradermally challenged with melanoma cells. a–c Experimental scheme (a) and tumor growth curves of mice challenged with B16F10-OVA (b) and B16F10-OTI (c) close to the vaccinated site. (d) Tumor growth curves of mice challenged at distant skin sites with B16F10-OVA (left) and B16F10-OTI (right). Data is representative of two independent experiments, n = 5 and n = 4 for DNA and protein vaccines, respectively. Bars are the mean ± SEM. ***p < 0.001, ****p < 0.0001 by Two-way ANOVA and Bonferroni post-hoc test.

Figure 3. Vaccination-induced skin Trm cells mediate strong protection against cutaneous melanoma independently of circulating CD8⁺ T cells. C57BL/6 mice were intravenously transferred with OVA-specific CD45.1⁺ OTI CD8⁺ T cells and the day later, were intradermally vaccinated with DNA-OVA or Protein-OVA. Control mice (CTRL) were vaccinated with empty plasmid (for DNA vaccination) or left unvaccinated (for protein vaccination). After 4–5 weeks, mice were depleted of circulating CD8⁺ T cells by administering anti-CD8 antibody and one week later, were intradermally challenged with melanoma cells. a–c Experimental scheme (a) and tumor growth curves of mice challenged with B16F10-OVA (b) and B16F10-OTI (c) close to the vaccinated site. (d) Tumor growth curves of mice challenged at distant skin sites with B16F10-OVA (left) and B16F10-OTI (right). Data is representative of two independent experiments, n = 5 and n = 4 for DNA and protein vaccines, respectively. Bars are the mean ± SEM. ***p < 0.001, ****p < 0.0001 by Two-way ANOVA and Bonferroni post-hoc test.

carrying OVA-specific Trm cell responses were completely protected. These results demonstrate that vaccination-induced skin resident and circulating memory compartments are sufficient to protect against melanoma tumors expressing a tumor-specific model antigen.

We next evaluated the anti-tumor role of vaccination-induced Trm cells in the context of a melanoma-associated self-antigen that involves CD8⁺ T cells bearing TCRs with lower affinity, which have been reported to inefficiently induce Trm cell responses in a viral infection model. Hence, mice were intradermally vaccinated with a plasmid encoding the melanocyte/melanoma antigen GP100, which is subject of peripheral tolerance. Interestingly, specific circulating and resident responses were efficiently elicited in DNA-GP100 vaccinated mice (Fig. 6a,b). Importantly, Trm cells suppressed the growth of B16F10 melanoma tumors in mice devoid of circulating CD8⁺ T cells (Fig. 6c). Tumor growth suppression was apparently at a greater extent in non-depleted mice, although this difference was not statistically significant and all mice developed tumors (Fig. 6c), further emphasizing the anti-tumor efficacy of Trm cells. The lack of fully protective responses in GP100-vaccinated mice may reflect the influence of mechanisms of central and peripheral tolerance, such as low TCR affinity and Treg-mediated regulation, respectively. We then sought to analyze the infiltration of GP100-specific Trm cells into melanoma tumors. Interestingly, CD90.1⁺ CD8⁺ T cells exhibiting distinctive CD69⁺ CD103⁺ phenotype were present in skin surrounding tumors and within tumors of GP100-vaccinated αCD8-depleted mice (Fig. 6d,e). In contrast, other CD8⁺ T cells infiltrating tumors did not display a Trm phenotype. Collectively, our results allow us to conclude that vaccination-induced Trm cells recognizing tumor-specific and self-antigens efficiently suppress melanoma growth and are highly desirable to achieve potent anti-tumor protection.

Discussion

During a long time, the existence of Trm cells was surprisingly ignored, considering that accumulating evidence supports that they provide superior protection and can be more abundant than their circulating counterpart. Their remarkable ability to mediate protective adaptive and innate immune responses has prompted the development of vaccination strategies that harness Trm cells to fight tumors. Here, we report that clinically applicable vaccination strategies efficiently establish protective Trm cell responses, unveiling a major contribution of the tissue-resident compartment to vaccination-induced immunity against melanoma. We demonstrated that a single intradermal administration of gene- or protein-based vaccines efficiently induces specific Trm cell responses against models of tumor-specific and self-antigens that protect against highly aggressive B16F10 melanoma. Tumor-specific Trm cells sufficiently and completely prevented the growth of melanoma, independently of circulating CD8⁺ T cells or other arms of the adaptive immunity elicited by intradermal vaccination, such as CD4⁺ T cells. To this end, we specifically dissected the contribution of vaccination-induced Trm cell responses by eliminating circulating CD8⁺ T cells through the administration of an αCD8 antibody at the memory phase of the response. This strategy enabled us to avoid the use of FTY720, which has been reported to interfere with the ability of T cells to infiltrate tumors, impair T cell function and even directly suppress tumor growth.

Total anti-tumor protection was observed in B16F10 models expressing either full length OVA or the immunodominant MHC class I-restricted OVA peptide; in the latter case, discarding the potential contribution of OVA-specific CD4⁺ T cells and antibody responses. We also confirmed the involvement of
Trm cells by delivering protein vaccines intraperitoneally, which bypasses the generation of skin-resident memory, while eliciting comparable levels of circulating memory CD8$^+$ T cells, compared to intradermal vaccination. Consistently, both vaccination routes prevented the growth of melanoma in non-depleted mice, but only intradermal vaccination effectively suppressed tumor growth in mice depleted of circulating CD8$^+$ T cells.

Interestingly, protective Trm cell responses were present in skin distant from the vaccinated site, which probably results from skin-wide seeding of Trm cell precursors expressing skin-homing molecules during the effector phase of the response, as has been observed for infection models. Additionally, skin Trm cells exhibit a rather persistent crawling behavior that allows them to continuously patrol the skin at a speed of ~2 mm/day. This persistent migration is essential for Trm cells to encounter their target cells and can further contribute to their dissemination through the skin. Importantly, we show that vaccination can efficiently rise skin-wide Trm cell responses from the endogenous T cell repertoire, which is pertinent in case these results are considered to be translated into a clinical setting.

We further validated the anti-tumor role of vaccination-induced Trm cell responses against a model of melanocyte-
To our knowledge, this is the first study to report that Trm cell responses against a self-antigen can be efficiently generated by vaccination. Interestingly, GP100 vaccination triggered the development of vitiligo, which has been shown to be necessary for the establishment of Trm cell responses. However, in our experimental setting, Trm cell responses can be efficiently elicited in the absence of vitiligo, at least with the OVA model. If vitiligo eventually promotes Trm cell formation induced by GP100 vaccination remains to be addressed. GP100-specific Trm cell responses drastically reduced the growth of B16F10 melanoma tumors in mice devoid of circulating CD8+ T cells, but in contrast to the OVA model, all vaccinated mice developed tumors. Moreover, anti-tumor protection was not significantly improved in non-depleted mice carrying both resident and circulating memory responses against GP100, emphasizing the protective nature of...
Trm cells. Incomplete tumor protection observed in this setting is probably a consequence of central and peripheral tolerance mechanisms that constrain responses against self-antigens. This is consistent with the weaker ability of pMEL-1 TCR-bearing CD8⁺ T cells to recognize the mouse GP100(25-33) peptide, as evidenced by reduced IFN-γ production in response to TCR stimulation. Interestingly, this model enabled us to identify tumor-infiltration of GP100-specific Trm cells displaying a distinctive CD69⁺CD103⁺ phenotype, which was not observed in the rest of tumor-infiltrating CD8⁺ T cells. Considering that our setting involved the depletion of the circulating memory, these results strongly suggest that vaccination-induced skin Trm cells can get positioned within tumors to fight melanoma. Collectively, our data demonstrate that vaccination-induced Trm cells mediate strong and skin-wide protection against cutaneous melanoma.

Vaccination is gaining great interest from the cancer immunotherapy field. Despite the limited success observed in initial trials, more effective vaccination platforms and the identification of antigens that drive tumor rejection have fostered the efficacy of cancer vaccines. In this context, gene- and protein-based vaccination used in this study has demonstrated safety and immunogenicity in different clinical trials. Both vaccination platforms can be used for either personalized vaccines targeting tumor-specific antigens resulting from missense mutations, or vaccines targeting highly prevalent tumor-associated self-antigens, which are suitable for broader use, including cancers with a low mutational load. Emerging evidence indicates that effective anti-tumor protection requires coordinated immunity across different tissues. Furthermore, the ability of CD8⁺ T cells to acquire a characteristic gene expression signature that enables them to become established in non-lymphoid tissues, is essential to defeat tumors. These recent findings are backed up by studies describing that tumor infiltration of CD8⁺ T cells displaying a Trm phenotype predicts better clinical outcomes in patients with different types of cancer. Hence, we conclude that cancer vaccines should evoke tissue resident memory responses to effectively protect against tumors.

**Methodology**

**Animals.** C57BL/6J wild-type (CD45.2), B6.Cg-Thy1 a/Cy Tg (TcraTcrrb)8Rest/J (pMEL-1), C57 BL/6-Tg(TcraTcrrb)1100Mjb/J (OTI) and CBy.SJL(B6)-Ptprc/J (CD45.1) mice were purchased from Jackson Laboratories, kept at the animal facility of Fundacion Ciencia & Vida and maintained according to the “Guide to Care and Use of Experimental Animals, Canadian Council on Animal Care”. This study was carried out in accordance with the recommendations of the “Guidelines for the welfare and use of animals in cancer research, Committee

**Figure 6.** Vaccination with a melanoma-associated self-antigen generates specific Trm cell responses and suppresses tumor growth. C57 BL/6 mice were intravenously transferred with GP100-specific CD90.1⁺ pMEL-1 CD8⁺ T cells and the day afterwards, intradermally vaccinated with a DNA vaccine encoding the melanoma-associated antigen GP100 (DNA-GP100). Control mice were vaccinated with empty pcDNA plasmid (CTRL). a-b GP100-specific memory responses were analyzed 4–5 weeks after vaccination in spleen (a) and skin (b) by flow cytometry. Representative dot-plots of CD90.1⁺ GP100-specific memory CD8⁺ T cells in total CD8⁺ T cell (a) and CD45⁺ live (b) populations. Memory phenotype and total number quantifications are also shown for each case. c-e Mice were either non-depleted or depleted of circulating CD8⁺ T cells by injecting anti-CD8 antibody (µCD8) 4–5 weeks after vaccination. One week later, mice were intradermally challenged with B16F10 melanoma cells. (c) Experimental scheme and tumor growth curves are shown for non-depleted (bottom left) and CD8⁺ T cell-depleted (bottom right) mice. (d-e) CTL and DNA-GP100 vaccinated mice depleted of circulating CD8⁺ T cells were sacrificed twelve-days after tumor challenge, and skin (d) and tumors (e) were analyzed by flow cytometry. Representative dot-plots of CD8 and CD90.1 expression in CD45⁺ live cells (left panels), and CD103 and CD69 expression in GP100-specific CD90.1⁺ CD8⁺ T cells (middle panel). In the case of tumor analysis, CD90.1⁺ (red dots) and CD90.1⁺ (black dots) CD8⁺ T cells were overlaid. Percentages of CD90.1⁺ CD8⁺ T cells for each quadrant are indicated. Quantification of total GP100-specific Trm cells, defined as CD3⁺CD8⁺CD90.1⁺CD103⁺CD69⁺ T cells are shown for each case (right panels). (a, b, d, e) Pooled data of two independent experiments, bars are the mean ± SEM, n = 6 mice per group. (c) Data is representative of two independent experiments, n = 3 mice per group. Bars are the mean ± SEM. *p<0.05, **p<0.01 for (a, b, d, e) by Mann-Whitney unpaired t test. *p<0.05 and **p<0.001 by Two-way ANOVA and Bonferroni post hoc test.
of the National Cancer Research Institute”. The protocol was approved by the “Committee of Bioethics and Biosafety” from Fundacion Ciencia & Vida. Blinding or randomization strategy were done whenever it was possible, no animals were excluded from the analysis and male and female mice were used indistinctly. Mice were allocated randomly in the different experimental procedures.

**Immunizations.** Mice were intradermally immunized in the lower back skin with 40 μg of the following vectors: pVAX-OVA (DNA-OVA), encoding the membrane bound form of chicken OVA; pcDNA-GP100 (DNA-GP100), encoding the melanoma antigen GP100; and empty pVAX and pcDNA plasmids were used as controls. Immediately, DNA electroperoration was performed by placing a parallel needle array electrode (two rows of four 2 mm pins, 1.5 × 4 mm gaps) over the injected blebs to deliver the electric pulses (two 1125 V/cm, 0.05 ms pulses followed by eight 275 V/cm, 10 ms pulses) using the Derma Vax™ DNA Vaccine Skin Delivery System (Cyto Pulse Sciences, Inc.). Plasmids were purified using the NucleoBond® PCI10000 EF (Macherey-Nagel, ref 740548) or the NucleoBond XtraMidi EF (Macherey-Nagel, ref 740420.50). In the case of protein vaccination (Protein-OVA), mice were intradermally or intraperitoneally immunized with 0.5 μg of an antibody targeting the C-type lectin receptor DEC-205 fused to the full-length OVA protein (αDEC-OVA) in combination with 25 μg of polyl:C (InvivoGen, ref 31852-29-6).

**Cells:** Mouse melanoma cell line B16F10 (ATCC CLR-6475) was obtained from American Type Culture Collection. B16F10-OVA cells were previously generated. B16F10-OTI×5-ZsGreen cells (B16F10-OTI) were generated by lentiviral transduction of B16F10 cell line with the pLVX-OT1 vector encoding the OVA257-264 (OTI) epitope minigene fused to ZsGreen. All melanoma cell lines were cultured in complete RPMI 1640 medium (ThermoFisher Scientific, ref 61870-036), supplemented with penicillin, streptomycin (ThermoFisher Scientific, ref 15140122), non-essential amino acids (ThermoFisher Scientific, ref 11140050), sodium pyruvate (ThermoFisher Scientific, ref 11360070) and heat-inactivated fetal bovine serum (ThermoFisher Scientific, ref 10437010) in a humidified incubator at 37°C with 5% CO2. All cell lines were routinely tested for mycoplasma contamination.

**Intravenous transfer of CD8+ T cells:** Naïve CD45.1+CD45.2+ OTI and CD90.1+ pMEL-1 TCR-transgenic CD8+ T cells were purified from secondary lymphoid organs of transgenic mice using the EasySep™ Mouse CD8+ T Cell Enrichment Kit (StemCell Technologies, ref 19853). Mice were intravenously injected with 2 × 10^3-3 × 10^5 cells in 100 μL of sterile PBS (ThermoFisher Scientific, ref 10010023).

**Flow cytometry staining:** Monoclonal antibodies specific for mouse molecules were purchased from Biologene: CD3-FITC (clone 17A2), CD3-APC (clone 17A2), CD3-PerCP/Cy5.5 (clone 17A2), CD8-Brillant Violet 421 (clone 53-6,7), CD45-PE (clone 30-F11), CD45-PerCP (clone 30-F11), CD45.1-PE/Cy7 (clone A20), CD45.1-FITC (clone A20), CD103-APC (clone 2E7), CD103-PerCP (clone 2E7), CD69-APC/Cy7 (clone H1.2F3), CD69-APC (clone H1.2F3), CD44-PerCP (clone IM7), CD62 L-PE (clone MEL-14), CD8β-FITC (clone YTS156.7.7), KLRF1-APC/Cy7 (clone 2F1/KLRG1), KLRF1-Brillant Violet 421 (clone 2F1/KLRG1), CXXCR3-PerCP/Cy5.5 (clone CXCR3-173), CD127-FITC (clone A7R34), PD-1-APC (clone 29 F.1A12), TruStain fcX (clone 93) and viability dye Zombie Aqua (ref 423101). E- and P-selectin ligands were detected by using hFc-E-selectin and hFc-P-selectin fusion proteins (R&D Systems, ref 575-ES, 737-PS), followed by staining with anti hIgG-APC antibody (clone HP6017). In the case of MHC-I multimer staining, PE-labelled H-2Kb/OVA257-264 dextramer (ImmuXed, ref JD2163) was used according to the manufacturer’s protocol. Samples were analyzed in a FACS-Canto II cytometer (BD Bioscience) and data analyzed using FlowJo version X.0.7 (Tree Star, Inc.).

**Intravascular staining:** Mice were injected intravenously with 3 μg of CD8β-FITC antibody (clone YTS156.7) in 300 μL of sterile PBS (ThermoFisher Scientific, ref 10010023), as previously described. Mice were euthanized 3 min after injection and blood, skin and lungs were analyzed by flow cytometry.

**CD8+ T cell depletion:** Mice were intraperitoneally injected with three 20 μg doses of rat monoclonal anti-CD8 antibody (BioXCell, clone YTS169.4, ref BE0117) in three consecutive days, 4–5 weeks after vaccination. Control mice were injected with the same amounts of rat IgG2b isotype-matched control anti-KLH (BioXCell, clone LT2, ref BE0090). Alternatively, mice were intraperitoneally injected with seven 25 μg doses of FTY720 (Sigma-Aldrich, ref SML0700) during consecutive days before CD8+ T cell analysis in blood.

**Preparation of tissue cell suspensions:** Spleens from vaccinated mice were mechanically disaggregated using microscope slides with ground edges (Sail Brand, ref 7105), and single-cell suspension was obtained using a 70 μm cell strainer (BD Falcon, ref 352350). For skin preparations, vaccinated and non-vaccinated skin was excised, cut in small fragments and digested in 1 mL non-supplemented RPMI 1640 medium (ThermoFisher Scientific, ref 61870-036) containing 5 mg/mL of collagenase type IV (Gibco, ref 17104019) and 5 μg/mL of DNase I (AppliChem, ref A3778,0010) for 30 min at 37°C. Skin pieces were then disaggregated using microscope slides with ground edges (Sail Brand, ref 7105), and single-cell suspension was obtained using a 70 μm cell strainer (BD Falcon, ref 352350) followed by a second digestion with 5 mL of supplemented RPMI 1640 medium containing 25 μg/mL of DNase I (AppliChem, ref A3778,0010) for 5 min on ice, to obtain cell suspensions. For lung preparations, lungs were excised, cut in small fragments and digested in 1 mL of non-supplemented RPMI 1640 medium (ThermoFisher Scientific, ref 61870-036) containing 5 mg/mL of collagenase type IV (Gibco, ref 17104019) and 5 μg/mL of DNase I (AppliChem, ref A3778,0010) for 60 min at 37°C, with shaking at 230 RPM. Blood was obtained by tail bleeding in 1.5 mL tubes containing heparin (Sanderson Laboratories), and red blood cells were lysed with RBC lysis buffer (Biologene, ref 420301).

**Tumor challenge:** Three days after the last dose of depleting antibody, 50 μL of PBS containing 1 × 10^6 of B16F10-OVA or B16F10-OTI melanoma cells were injected intradermally in the lower back near the vaccination site or at a distant site in the upper back. Tumor growth was monitored by measuring perpendicular tumor diameters with calipers. Tumor volume was calculated using the following formula:

\[ V = \frac{1}{2} \times (x \times y \times z) \]

where V is the volume, x, y, and z are the three perpendicular diameters.
formula: \[ V = \frac{(D \times d^3)}{2} \] where \( V \) is the volume (\( \text{mm}^3 \)), \( D \) is the larger diameter (\( \text{mm} \)) and \( d \) is the smaller diameter (\( \text{mm} \)). Mice were sacrificed when moribund or when the mean tumor diameter was \( \geq 15 \text{ mm} \), according to the approved ethical protocol.

**Statistical analysis:** Statistical analysis was performed using Graphpad Prism software (Graphpad Software Inc.). Unpaired t tests were performed pairwise between relevant groups. In the case of tumor growth, Two-way ANOVA was performed between relevant groups. Error bars in Figs. indicate the mean plus SEM. \( P \) value < 0.05 was considered statistically significant; \( *P < 0.05, **P < 0.01, ***P < 0.001 \) and \( ****P < 0.0001 \).

**Disclosures of potential conflicts of interest**

There are no conflict of interest or financial disclosures between authors.

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